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GUANINE NUCLEOTIDES MODULATE CELL SURFACE cAMP-BINDING SITES IN MEMBRANES FROM *DICTYOSTELIUM DISCOIDEUM*

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D.discoideum contains kinetically distinguishable cell surface cAMP binding sites. One class, S, is slowly dissociating and has high affinity for cAMP (K_d = 15 nM, $t_{\frac{1}{2}}$ = 15 s). A second class is fast dissociating ($t_{\frac{1}{2}}$ about 1 s) and is composed of high affinity binding sites H ($K_d \approx 60$ nM), and low affinity binding sites L ($K_d = \approx 450$ nM) which interconvert during the binding reaction. Guanine nucleotides affect these three binding types in membranes prepared by shearing *D.discoideum* cells through Nucleopore filters. The affinity of S for cAMP is reduced by guanine nucleotides from 13 nM to 25 nM, and the number of S-sites is reduced about 50%. The number of fast dissociating sites is not altered by guanine nucleotides, but these sites are mainly in the low affinity state. Half-maximal effects are obtained at about 1 μM GTP, 2 μM GDP and 10 μM Gpp(NH)p(guanyl-5'-yl-imidodiphosphate); ATP and ADP are without effect up to 1 mM. These results indicate that *D.discoideum* cells have a functionally active guanine nucleotide binding protein involved in the transduction of extracellular cAMP signals via cell surface cAMP receptors.

cAMP has a hormone-like action in the cellular slime molds. Extracellular cAMP is involved in chemotaxis-mediated cell aggregation (1), morphogenesis of a multicellular structure into a fruiting body (2), and cell-differentiation in the multicellular organism (3). cAMP is detected by cell surface receptors and induces several intracellular responses, such as the activation of guanylate and adenylate cyclase (reviewed in 4-6). The produced intracellular cGMP is involved in the cAMP induced chemotactic reaction (7), while the produced intracellular cAMP is subsequently secreted by which the cAMP signal is relayed (8).

The mechanism of signal transduction from cell surface cAMP receptors to the cyclases is far from understood. It has been shown that an adaptation process takes place during stimulation with cAMP, and that this adaptation process is localized between cell surface cAMP receptors and the two cycla-

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ses (9-10). Adaptation of adenylate cyclase is essential for the unidirectional relay of the cAMP signal in a field of aggregating cells (11), while adaptation of guanylate cyclase seems to be essential during chemotaxis (12). It is evident that the target of the occupied cell surface cAMP receptor must be identified in order to understand the mechanism of signal transduction and adaptation.

Kinetic binding experiments on a second time-scale have recently revealed the existence of three kinetically different cell surface cAMP-binding sites on intact *D.discoideum* cells (13). A small class is slowly dissociating and has high affinity for cAMP (this class was designated as S; K_d = 15 nM, $t_{\frac{1}{2}}$ = 15 s). The majority of the binding sites release bound cAMP very fast ($t_{\frac{1}{2}} \approx 1$ s). This class contains a high affinity and a low affinity component (designated respectively as H and L; $K_d \approx 60$ nM and $K_d \approx 450$ nM respectively). During the binding reaction there is a time and cAMP-dose dependent transition of these high to low affinity binding components (13).

An analogous transition of high affinity binding to low affinity binding of hormones such as β-adrenergic agonists and chemotactic peptides to mammalian cells appears to be due to the coupling of a high affinity receptor to a guanine nucleotide binding protein (N-protein) and the subsequent activation of adenylate cyclase (14-17). In *D.discoideum* the existence of a N-protein has been proposed based on the cholera toxin-induced ADP-ribosylation and GTP affinity labelling of a 42 kD protein which is very similar to the N-protein of mammalian cells (18). However, experiments on a functionally active N-protein in *D.discoideum* have yielded negative results until now (adenylate cyclase is not activated in vitro by NaF, GTP, or forskolin (6,19,20).

In this report membranes of *D.discoideum* have been prepared which still contain the three cAMP-binding types H, L, and S. The effects of guanine nucleotides on these binding types demonstrate the presence of a functionally active N-protein in *D.discoideum* with a mode of action similar to the N-protein in mammalian cells.

Materials and Methods

D.discoideum, NC4(H), was grown, harvested and starved in suspension for 5 h, and binding of [^{3}H]cAMP to cells was measured as described earlier (13). Membranes were prepared by shearing the cells (21). Cells starved for 5 h were washed twice with 10 mM KH2PO4/Na3HPO4, pH 6.5, and resuspended in this buffer at a density of $2x10^8$ cells/ml. Cells were aerated at 20°C for 10 min. Four ml were pressed through a Nucleopore filter at 0°C (diameter 25 mM, pore size 5 µm). The filter was washed with 1 ml buffer, and the lysate was centrifuged for 5 min at 10,000xg in a small swing-out rotor. The supernatant was removed and the pellet was resuspended in 8 ml buffer. The membranes were kept on ice during the experiments which were completed within 1 h after membrane preparation. Binding of [^{3}H]cAMP to membranes was measured at 20°C in an incubation volume of 100 µl containing different concentrations [^{3}H]cAMP (1.5 TBq/mmole), nucleotides and 80 µl membranes. Bound [^{3}H]cAMP was separated from free [^{3}H]cAMP by centrifugation of 95 µl of the incubation mixture through 200 µl silicon oil (AR20/AR200 = 2/1) in a homemade swing-out rotor at 10,000 x g for 30 s (13).

Results

The association of 30 nM [^{3}H]cAMP to aggregative *D.discoideum* cells at 20°C is shown in Fig. 1A. Binding of [^{3}H]cAMP rapidly increases, reaching a maximum at about 6 s which is followed by a decline of [^{3}H]cAMP-binding to a steady state value obtained at about 45-60 s. Previously (13) it has been demonstrated that this decline is neither due to degradation of [^{3}H]cAMP, nor to dilution of [^{3}H]cAMP by secreted cAMP, nor to a reduction of the number of cAMP-binding sites. The reduction of binding between 6 s and 45 s, is due to a decrease of the affinity of the cAMP binding sites. The association of 30 nM [^{3}H]cAMP to membranes is slightly slower than to cells (Fig. 1B), and the overshoot observed in cells is absent in the membranes with or without guanine nucleotides. Gpp(NH)p (guanyl-5'-yl-imidodi-

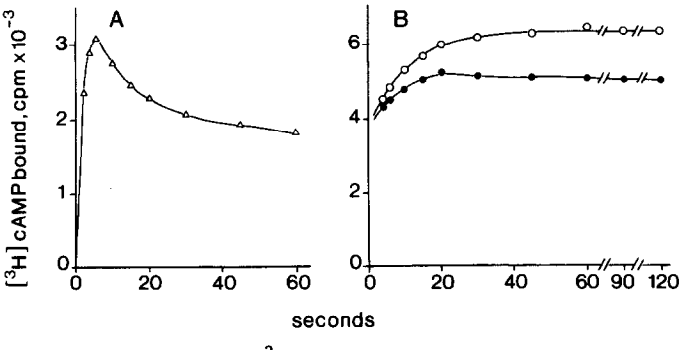

Fig. 1. Association of 30 nM [^{3}H]cAMP to aggregative *D.discoideum* cells (A) and to membranes (B) in the absence (○) or presence (●) of 100 µM Gpp(NH)p.

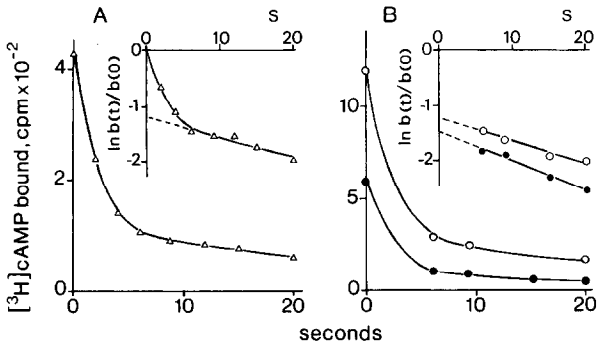

Fig. 2. Dissociation of [^{3}H]cAMP from cells (A) and membranes (B). Cells or membranes were pre-incubated in the absence (Δ, ○) or presence (●) of 100 μM Gpp(NH)p during 45 s (A) or 75 s (B). Then, at t = 0 s, 2 μl 10^{-2}M cAMP was added and cell-associated radioactivity was determined at the times indicated. Insets: semi-logarithmic plot of cell associated radioactivity as % of equilibrium binding.

phosphate) at 100 μM inhibits the equilibrium binding of 30 nM [^{3}H]cAMP by about 20%.

The dissociation of 2 nM [^{3}H]cAMP from the receptors was measured by the addition of excess cAMP. On intact cells (Fig. 2A) two components with different off-rates were observed. About 70% of the [^{3}H]cAMP was bound to a fast dissociating site with a half-life of about 1.7 s (13), while the remaining [^{3}H]cAMP was bound to a slowly dissociating site with a half-life of about 15 s. These fast and slowly dissociating sites are also present on membranes prepared from these cells (Fig. 2B). Gpp(NH)p at 100 μM inhibits the equilibrium binding of 2 nM [^{3}H]cAMP by about 53%. The proportioning of fast and slow dissociating sites is not strongly altered by Gpp(NH)p, neither is the off-rate (inset Fig. 2B).

Previously (13) we have shown that the decrease of [^{3}H]cAMP-binding between 6 and 45 s in intact cells (Fig. 1A) is a property of the fast dissociating binding sites, and is caused by the transition of a high affinity binding type (designated as H; $K_d \approx 60$ nM, $k_{-1} = 0.5\ s^{-1}$) to a low affinity binding type (designated as L; $K_d \approx 450$ nM, $k_{-1} = 1\ s^{-1}$). The slowly dissociating binding sites are occupied with [^{3}H]cAMP more or less independent from the fast dissociating sites (13). This type has been designated as S ($K_d = 15$ nM, $k_{-1} = 0.05\ s^{-1}$).

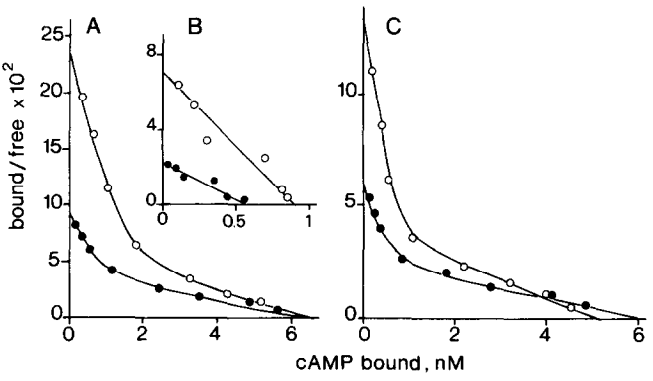

Fig. 3. Scatchard plots of cAMP-binding to membranes of *D.discoideum* in the absence (○) or presence (●) of 100 µM Gpp(NH)p. A. Binding to all three sites was measured at different cAMP concentrations after an incubation period of 75 s. B. Binding to S was measured by incubating with different [^{3}H]cAMP concentrations during 75 s followed by a 6 s chase with excess cAMP (which releases all [^{3}H]cAMP bound to H and L). C. Binding to H + L was calculated by subtraction of S-specific binding at the moment of the cAMP chase from total equilibrium binding. The rate constant of dissociation of S (k_{-1} = 0.05 s^{-1}) predicts that 25% of the occupied S-sites dissociate during 6 s. Therefore, S-specific binding at the onset of the cAMP chase is 1.33 fold the binding after the 6 s chase.

The effect of Gpp(NH)p on the three binding types mentioned above is demonstrated in Scatchard plots (Fig. 3). Gpp(NH)p inhibits equilibrium binding only at low [^{3}H]cAMP concentrations (Fig. 3A). The total number of binding sites is not altered significantly by Gpp(NH)p. These data represent the equilibrium binding to the sum of H, L, and S. The specific binding to S is measured by a 6 s chase with excess of cAMP, by which all [^{3}H]cAMP bound to H and L is released. Gpp(NH)p affects the binding to S in two ways; the number of S binding sites is reduced about 50%, and the affinity of S for cAMP is reduced from K_d = 13 nM to K_d = 25 nM (Fig. 3B). Binding of [^{3}H]cAMP to the sum of H and L is calculated by subtraction of S-specific binding at the moment of the cAMP chase from total binding. The result shows (Fig. 3C) that the Scatchard plot is more linear in the presence of 100 µM Gpp(NH)p with an apparent dissociation constant of 275 nM, which resembles the dissociation constant of L on intact cells. Therefore, it is concluded that in the presence of Gpp(NH)p the fast dissociating sites are mainly in the low affinity state.

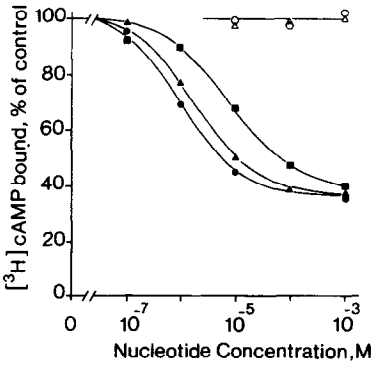

Fig. 4. Nucleotide specificity of the inhibition of the binding of 2 nM [^{3}H]cAMP to membranes from *D.discoideum* after an incubation period of 75 s. (●), GTP ; (▲), GDP; (■), Gpp(NH)p; (○), ATP; (△), ADP.

The nucleotide specificity of the inhibition of the equilibrium binding at 2 nM [^{3}H]cAMP is shown in Fig. 4. GTP, GDP, and Gpp(NH)p yield approximately the same maximal inhibition, while ATP and ADP are inactive. A half-maximal effect is obtained at about 1 μM GTP, 2 μM GDP, and 10 μM Gpp(NH)p.

Discussion

The membranes prepared by shearing of the cells by pressing them through a Nucleopore filter have approximately the same heterogeneity of cAMP-binding types as are present on intact cells. Guanine nucleotides alter the affinity and number of the slowly dissociating binding sites. Fast dissociating cAMP-binding sites are composed of high and low affinity components, which interconvert on intact cells in a time and cAMP-dose dependent manner. In the presence of guanine nucleotides almost all fast dissociating sites are in the low affinity state. The nucleotide specificity of the inhibition of cAMP binding strongly suggests that the effect is mediated by a guanine nucleotide binding protein (N-protein), which, in mammalian cells, is involved in the regulation of hormone dependent adenylate cyclase activity (14-17).

The interactions of cAMP and N-protein with the fast dissociating cAMP-binding sites in *D.discoideum* are almost identical to the interactions of β-adrenergic agonists and N-protein with β-adrenergic receptors on erythro-

cytes. At first, β-adrenergic receptors are in a high affinity conformation and transfer to a low affinity conformation by the interaction with β-adrenergic agonists (14). The low affinity conformation can also be induced in membranes by the addition of guanine nucleotides, which is due to the coupling of the occupied high affinity receptor with a guanine nucleotide containing N-protein (15). The low affinity receptor-N-protein complex then activates adenylate cyclase (16). It is tempting to suggest that cAMP-mediated activation of adenylate cyclase in *D.discoideum* proceeds via the same mechanism. Although the present results indicate a functional coupling of an N-protein with cell surface cAMP-receptors, no evidence for such a coupling of N-protein with adenylate cyclase exists; drugs known to interfere with this coupling in mammalian cells, such as NaF, GTP or forskolin, do not affect adenylate cyclase in *D.discoideum*. In addition, activation of adenylate cyclase by cAMP in vitro has not been observed in *D.discoideum*, which may indicate that adenylate cyclase is functionally uncoupled from the N-protein in the membrane preparations used previously (6,19,20).

The function of the slowly dissociating cAMP-binding sites is less evident. The observation that the association of cAMP to this binding site is more or less independent from the association and interconversion of the fast dissociating binding sites H and L (13) may indicate that binding to S mediates a different response of the cells to cAMP, such as the activation of guanylate cyclase or the entrance of Ca^{++}.

Nevertheless, the present results suggest that the first event after activation of cell surface cAMP receptors by cAMP has been identified as the interaction of cAMP receptors with an N-protein. Further experiments on the kinetics of this interaction and on the coupling of N-protein with adenylate and guanylate cyclase may shed light on the complex adaptation processes which occur in *D.discoideum* at a locus between cell surface receptors and adenylate or guanylate cyclase.

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